

## EFFECT OF pH UPON PROTEOLYSIS BY PAPAIN\*

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The existence of pH optima for proteolysis was early established, primarily through measurements of the initial rate of reaction as a function of pH. When synthetic substrates for proteolytic enzymes were discovered, it was found that the rate of digestion depended upon the amino acids of the peptide and their arrangement in the chain. We have investigated the effect of pH upon the hydrolysis of peptide bonds of different specific rates of digestion in an endeavor to find out whether a differential effect of pH upon the extent of enzymic hydrolysis of proteins could be demonstrated.

The evidence available in the literature was indirect, but was consistent with such a pH effect. The optimum pH for the initial digestion of different proteins by papain varies, being about 5 for the digestion of gelatin and 7 for casein (1) and egg albumin (2). Lineweaver and Schwimmer (1) found a similar pH-activity function for the action of both crystalline and commercial papain on casein. The initial rate of digestion at pH 5 and 9 was about 60 per cent of that at pH 6.5 to 7.0. This was true both when the casein was in suspension in the isoelectric region and when it was held in solution by urea. Bergmann and coworkers used pH 5 throughout in their study of the digestion of synthetic substrates by papain. Rocha e Silva and Andrade (3) obtained evidence of two pH optima, about 5 and 7, for the action of papain upon benzoylargininamide. Pepsin digests synthetic substrates less rapidly at its normal pH optimum of about 2 than at pH 4.2 (4, 5). The latter pH was used successfully by Pope for the purification of diphtheria antitoxin by pepsin (6).

We have investigated the effect of pH upon the course of proteolysis by papain, using two substrate systems: (a) the synthetic substrates of Bergmann and coworkers, in which a first order reaction can be demonstrated and (b) casein. The rate and extent of proteolysis of casein at pH 7, 5, and 2.5 were determined. An optimum pH of about 5 for the "peptidase" action of papain was demonstrated in both systems. The extent of hydrolysis of casein is far greater at pH 5 than at the optimum for initial hydrolysis, about pH 7.

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## EXPERIMENTAL

*Enzyme*—Commercial papain was purified by precipitation with 70 per cent methanol essentially according to Irving, Fruton, and Bergmann (7). The final precipitate was dried by sublimation of the water. After it was equilibrated with the moisture of the laboratory atmosphere, it contained 12.81 per cent N (Kjeldahl), 10.72 per cent protein N (Kjeldahl, trichloroacetic acid precipitation), and had about the same activity as the product obtained by the above workers.

*Synthetic Substrate*—Hippurylamide (8), benzoyl-*l*(+)-argininamide (9), and N-carbobenzoxy-*l*(+)-isoglutamine (10) were synthesized. In each case the melting point and nitrogen content (Kjeldahl) checked with the value in the reference.

*Proteolysis of Synthetic Substrates*—The methods of Bergmann and co-workers were followed throughout. Hydrolysis was measured by the Grassmann-Heyde titration (11). The digestion of these synthetic substrates by cysteine-papain is a first order reaction, and the specific rate of digestion is expressed (12) by  $C_{\text{substrate}} = k_1$  per mg. of enzyme N. The air-dry enzyme was weighed directly and enzyme nitrogen calculated on the basis of the protein N content determined. Substrate-buffer mixture, 0.2 M citrate final concentration, was adjusted to the correct pH with 1 N HCl or NaOH and made to volume. Relatively high enzyme concentrations, 0.2 to 0.3 mg. of protein N per ml., were employed in order to obtain adequate rates of digestion at the extremes of the pH range. All digestions were made at 40°.

*Casein Substrate*—Commercial casein, 14.1 per cent N, air-dry, 15.3 per cent N on a moisture-free basis, was dissolved in citric acid or NaOH to the desired pH and made up to a 3 per cent solution, based on the air-dry weight, with buffer. The buffer concentration tabulated is the molarity of the final solution: pH 2.5, 0.2 M (citric acid-citrate) (This was the maximum buffer concentration obtainable. The buffer capacity was not sufficient to prevent entirely a drift of pH, about 0.2 unit in 24 hours and 0.3 in 96 hours.); pH 5.0, 0.3 M (citric acid-NaOH) (suspension of finely ground casein); pH 7.0, 0.075 M ( $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ ); pH 9.5, 0.1 M ( $\text{Na}_2\text{CO}_3\text{-Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$ ); pH 10.5, 0.1 M ( $\text{Na}_2\text{CO}_3\text{-Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$ ).

*Proteolysis of Casein*—The enzyme was activated for 1 hour at pH 7 with an equal weight of cysteine. An *E:S* (enzyme to substrate) ratio of 1:30, calculated on the protein N basis, was used in the experiments presented here. Digestion was carried out at 40° under toluene. Proteolysis was measured by the Van Slyke nitrous acid method (5 minute reaction time) and the formol titration. Results by the two methods agreed well, except in the later stages of the digestion, in which the values by formol titration ran ahead of those by the Van Slyke method, presumably owing to the re-

lease of proline. The data plotted were obtained by the Van Slyke method. The release of free amino acids was followed by the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (13).

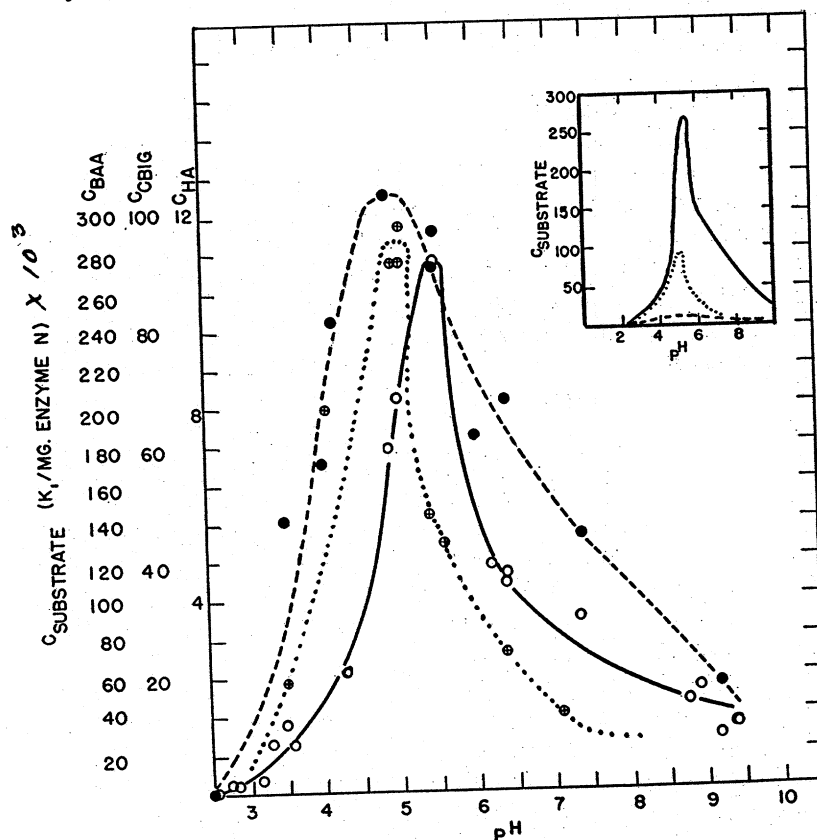


FIG. 1. Effect of pH on the digestion of benzoylargininamide (BAA, O), carbobenzoxy-*l*(+)-isoglutamine (CBIG,  $\oplus$ ), and hippurylamide (HA,  $\bullet$ ) by papain.

### Results

The three synthetic substrates employed were benzoylargininamide (BAA), carbobenzoxy-*l*(+)-isoglutamine (CBIG), and hippurylamide (HA). The first has a free basic group in the side chain, the second has an acid group, and the last has neither. Our enzyme preparation digested these at relative rates of about 20:8:1. When adjusted for the difference in specific activity, the three curves (Fig. 1), show that the pH function of the hydrolysis is quite similar for the three substrates. The optimum pH for the BAA is about 0.5 unit higher than those for the other two substrates.

HA has a slightly broader curve than the two more readily digestible substrates. The normal plot of activity *versus* pH is shown in the insert of Fig. 1 for comparison. Rocha e Silva and Andrade (3) obtained a second lower maximum in the curve at pH 7 for the hydrolysis of BAA and CBIG with papain prepared from dried latex by essentially the same procedure we used for purifying the commercial enzyme. They also reported evidence that the relative activity of two different papain preparations upon those two substrates was different, indicating the presence of two different peptidases. In our experiments a clearly defined pH optimum is found at pH 5 to 5.5 for hydrolysis of each of the three dissimilar peptides.<sup>1</sup>

Preliminary experiments had indicated that the extent of hydrolysis of casein by papain was decreased by an unfavorable pH. These results were confirmed, but when the data were replotted to take into account the much slower over-all rate at an unfavorable pH, the curves obtained at pH 7 and 2.5 were close together, indicating no change in specificity of hydrolysis. To obtain more satisfactory data upon this point, experiments were carried out at pH 7.0, 5.0, and 2.5 under conditions otherwise comparable. We were not able to obtain comparable data in the alkaline region, for at pH 9.5, 10.5, and above, the enzyme was irreversibly inactivated within a few hours incubation at the reaction temperature of 40°. Control experiments at pH 2.5 and 7.0, in which the digest was adjusted to pH 5 after 100 to 150 hours digestion, showed that the enzyme was not destroyed at these pH values. The controls carried the digestion substantially to the same point that was reached by the sample at pH 5.

The course of the reaction at pH 2.5 was quite similar to that at pH 7 (Fig. 2). The two curves can be practically superimposed by adjusting the abscissa scale for the greater rate of digestion at pH 7 (5-fold). The course of the reaction at pH 5 is different. The initial rate of reaction is slightly slower than at pH 7 (as previously shown by Lineweaver and Schwimmer), but it does not level off until the digestion has proceeded about twice as far as at pH 7. Proteolysis was still proceeding at a low but definite rate when the experiments were terminated.

When the data of Fig. 2 were plotted, for convenience, upon a logarithmic time scale, a linear relationship for the extent of digestion *versus*  $\log t$  was observed over the range 1 to 100 hours and beyond at pH 2.5 and 7.0. Examination of other data, ours and in the literature, showed that the same plot was applicable. The data of Lineweaver and Schwimmer ((1) Fig. 5, Curve 10) obtained at pH 7, and of Leipert and Häfner ((14) Table I) at pH 5 show the same relationship. The physical significance of such a plot is obscure, for it obviously is not applicable as zero time is approached.

<sup>1</sup> Dr. Joseph S. Fruton has observed this additional pH optimum at 7.5 with certain papain preparations, but not with others. (Personal communication.)

Evidence of a different type was also obtained in the experiments plotted in Fig. 2. The rate of appearance of free amino acids relative to that of amino nitrogen was determined. The latter data were taken from the digestion curve at the time the free amino acids were determined. Complete hydrolysis of casein to amino acids releases 71 per cent of the total nitrogen as  $\alpha$ -amino N determinable by the Van Slyke reaction (calculated from the data of Chibnall (15)), and 75 per cent of the total nitrogen as measured by the ninhydrin reaction (13). Calculation (Table I) of the free peptide

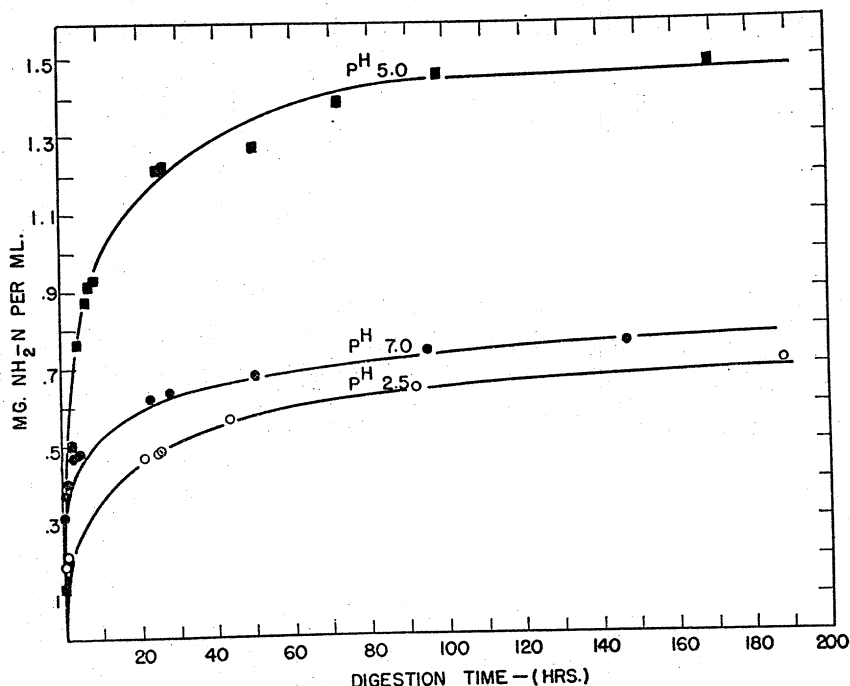


FIG. 2. Release of amino nitrogen by papain digestion of casein at pH 2.5, 5.0, and 7.0.

amino N ( $E$ ) and average number of amino acids per peptide ( $F$ )<sup>2</sup> shows that there is an initial rapid production of peptides, consisting, on the average, of about 5 to 6 amino acid residues. This is followed by increasing production of free amino acids. The relative rate of production of amino acids to amino N increases as the digestion proceeds; at pH 5 amino acids are being released at twice the rate of production of amino N during the

<sup>2</sup> The non-reactivity of proline N in the Van Slyke amino N determination does not affect this calculation very much, for in the equation  $F = D/E = A - C/B - C$ , both  $A$  and  $B$  are affected.

latter stage of the digestion. Such a result can most readily be ascribed to the hydrolysis of dipeptides. The average peptide length increases slightly because of this removal of peptides which are shorter than the average. The final products correspond to the production of two free amino acids and one tetrapeptide for each 6 amino acid residues initially present.

The results at pH 7 and 2.5 are strikingly similar; although the rate of digestion at pH 2.5 is one-fifth that at pH 7, the relative rates of production

TABLE I  
*Production of Peptides and Free Amino Acids by Papain Digestion of Casein*  
3 per cent solution containing 3.01 mg. per ml. of potential amino nitrogen (A)  
and 3.19 mg. per ml. of potential free amino acid nitrogen (A').

pH	Time	Amino N released (B)	Free amino acid N released (C)	Potential amino N in peptides (D) = (A - C)	Free amino N in peptides (E) = (B - C)	Average No. of amino acids in peptides (F) = (D/E)	Peptide bonds split (G) = $(100 \times B/A)$	Amino acids released (H) = $(100 \times C/A')$
	hrs.	mg. per ml.	mg. per ml.	mg. per ml.	mg. per ml.		per cent	per cent
5.0	0.42	0.13		3.01	0.13	23.3	4.3	
	0.75	0.38		3.01	0.38	7.9	12.6	
	3	0.65	0.06	2.95	0.59	5.0	21.6	1.9
	7	0.91	0.16	2.85	0.75	3.8	30.2	5.0
	25	1.21	0.45	2.56	0.76	3.4	40.2	14.1
	72	1.39	0.66	2.35	0.73	3.2	46.2	20.7
	169	1.49	0.87	2.14	0.62	3.5	49.5	27.3
	266	1.55	0.99	2.02	0.56	3.6	51.5	31.0
	0.65	0.31		3.01	0.31	9.7	10.3	
	1.03	0.37		3.01	0.37	8.1	12.3	
7.0	3.14	0.47		3.01	0.47	6.4	15.6	
	28.5	0.63	0.10	2.91	0.53	5.5	20.9	3.1
	50	0.68	0.20	2.81	0.48	5.9	22.6	6.3
	182	0.78	0.44	2.57	0.34	7.6	25.9	13.8
	1.42	0.21		3.01	0.21	14.3	7.0	
	21	0.47	0.05	2.96	0.42	7.0	15.6	1.6
2.5	48	0.59	0.15	2.86	0.44	6.5	19.6	4.7
	119	0.65	0.17	2.84	0.48	5.9	21.6	5.3
	188	0.71	0.22	2.79	0.49	5.7	23.6	6.9

of amino nitrogen and amino acids are essentially the same. At pH 5 the concentration of peptides is higher but not enough to account for the much greater rate of production of amino acids on that basis alone. It is apparent that a peptidase action (as measured by increase in free amino acids and lower peptide length), which is quite slow at pH 7, is rapid at pH 5. These results are consistent with those obtained on the synthetic peptides.

The initial stage of the digestion of casein by papain was studied in the following experiment. 150 gm. (air-dry) of casein were digested at pH 7 by

300 mg. (air-dry) of purified papain, activated by thioglycolic acid. After 3 hours incubation at 40°, the extent of digestion was 0.95 per cent. 20 ml. of superoxol were added, and the solution was heated to 95° and filtered. The solution was cooled and adjusted to pH 4.6 with acetic acid. The precipitated protein was filtered off, washed, and air-dried. The yield was 126.5 gm., or 83 per cent on a moisture-free basis.

The precipitated protein was examined electrophoretically by R. C. Warner of this Laboratory, who found (pH 7.8, veronal buffer) that the boundary of  $\beta$ -casein, which constitutes 20 per cent of casein (16), was essentially absent. A diffuse boundary with a mobility of 5.5 cm. volt<sup>-1</sup> sec.<sup>-1</sup> was present, apparently replacing the normal  $\alpha$ -casein peak, mobility of 6.3 cm. volt<sup>-1</sup> sec.<sup>-1</sup>. Electrophoretic examination of a control sample treated similarly, except for the omission of the enzyme, showed the normal  $\alpha$ - and  $\beta$ -casein pattern. Thus digestion of about 1 per cent of the peptide bonds present had eliminated the  $\beta$ -casein fraction from the acid-precipitable protein and made the remaining portion definitely less homogeneous. The close correlation between the loss of acid-precipitable protein (17 per cent) and the amount of  $\beta$ -casein initially present (20 per cent) is consistent with the electrophoretic pattern.

#### DISCUSSION

The results cited are, so far as we are aware, the first demonstration that the optimum pH for the initial rate of enzymic digestion of a protein is not that for its complete digestion. It is probable that other proteins which have an optimum for initial digestion of about pH 7 will be digested more completely at pH 5. In fact, Willstätter, Grassmann, and Ambros (17) found an optimum pH of about 5 for the digestion of egg albumin peptone by papain, and Calvery (18) obtained hydrolysis of two-thirds of the peptide bonds of egg albumin by papain at pH 5. Analogously, pepsin might be expected to digest proteins further at about pH 4, the optimum for its action on peptides, than at pH 2, at which the initial rate of digestion of proteins is highest.

Lineweaver and Schwimmer (1) obtained about 25 per cent hydrolysis of casein by papain at pH 7. They note that Leipert and Häfner (14) reported release of 47 per cent of the total nitrogen of casein by papain at pH 5, and call attention to the possibility that the difference between the two results may be due to the different pH employed. It is not clear whether Leipert and Häfner subtracted the  $\epsilon$ -amino nitrogen initially present in calculating their results; if they did not, their results are in fair agreement with ours at the same pH. It should be noted that hydrolysis was still proceeding at the termination of our experiments. Calvery (18) found it necessary to use

rather high  $E:S$  ratios and long reaction periods to obtain definite end-points for proteolysis.

Winnick (19) digested casein with several enzymes and partly characterized the split-products. Papain was used at pH 7.5, and the results, when recalculated to our method of expressing the data, are in close agreement with ours. Using an  $E:S$  ratio of 1:200, he obtained hydrolysis of 19.7 per cent of the peptide bonds and release of 1.7 per cent of the free amino acids in 36 hours at 40°. His experiments were concluded at this point, when the production of free amino acids was just becoming appreciable.

The data of Table I show that the rate of digestion of various peptide bonds in casein is differentially affected by a change in pH. The possibility of producing large peptide fragments by such a mechanism does not appear promising, for even at pH 2.5 the digestion proceeds rapidly to the level of hexapeptides. This is a number average, and there probably are higher peptides present, but if the splitting is at all symmetrical, the amount of large polypeptides must be rather small.

Winnick and Greenberg (20) have recently proposed that proteinases be described as "acido-," "baso-," or "neutroproteinases," depending upon the optimum pH for their activity. Our results indicate that papain would fall into different classifications in this system, according to the extent of digestion at which the measurements were made. It is also doubtful whether there are sufficient data upon other proteinases for such a scheme to be established upon a sound basis.

The synthesis of the synthetic substrates by Lieutenant (j.g.) David G. Doherty, U. S. N. R., and his cooperation in the initiation of this work are gratefully acknowledged.

#### SUMMARY

1. At pH 5 papain rapidly hydrolyzes approximately 50 per cent of the peptide bonds of casein, releasing 30 per cent of the amino acids as free amino acids. Under comparable conditions at pH 7 only 25 per cent of the peptide bonds are split, although the latter pH is the optimum for the *initial* rate of digestion.
2. The digestion is characterized by a rapid production of peptides, averaging 4 to 6 units, followed by the release of amino acids without much change in the average length of the peptides present.
3. The course of the hydrolysis at pH 7 is similar to that at pH 2.5, as judged by the relative rates of production of amino nitrogen and free amino acids, although the rate of reaction at pH 7 is 5 times that at pH 2.5.
4. The effect of pH upon hydrolysis by papain of benzoylargininamide, carbobenzoxy-*L*-isoglutamine, and hippurylamide, which are digested at



relative rates of 20:8:1, was measured. The three curves are similar when adjusted for the difference in specific rates of digestion; the optimum pH for benzoylargininamide is approximately 5.5 and for the other two, 5.0. The optimum pH for the action of papain upon synthetic peptides is thus comparable to that for its action upon casein split-products.

5. Hydrolysis of about 1 per cent of the peptide bonds of casein by papain eliminated the  $\beta$ -casein fraction from the digestion products precipitable at pH 4.7, but the  $\alpha$ -casein fraction appeared to be less homogeneous and of altered mobility.

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